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N-glycosylation of human acetylcholinesterase: effects on activity, stability and biosynthesis

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The role of N-glycosylation in the function of human acetylcholinesterase (HuAChE) was examined by site-directed mutagenesis (Asn to Gln substitution) of the three potential N-glycosylation sites Asn-265, Asn-350 and Asn-464. Analysis of HuAChE mutants, defective in a single or multiple N-glycosylation sites, by expression in transiently or stably transfected human embryonal 293 kidney cells suggests the following. (a) All three AChE glycosylation signals are utilized, but not all the secreted molecules are fully glycosylated. (b) Glycosylation at all sites is important for effective biosynthesis and secretion; extracellular AChE levels in mutants defective in one, two or all three sites amounted to 20–30 %, 2–4 % and about 0.5 % of wild-type level respectively. (c) Some glycosylation mutants display impaired stability, as reflected by increased susceptibility to heat inactivation; substitution of Asn-464 has the most pronounced effect on thermostability. (d) Abrogation of N-glycosylation has no detectable effect on the enzyme activity of HuAChE; all glycosylation mutants, including the triple mutant, hydrolyse acetylthiocholine efficiently, displaying K_m, $k_{cat.}$ and $k_{cat.}/K_m$ values similar to those of the wild-type enzyme. (e) In most mutants, inhibition profiles with edrophonium and bisquaternary ammonium ligands are identical with those of wild-type enzyme; the Asn-350 mutants, however, exhibit a slight decrease in their affinity towards these ligands. (f) Elimination of oligosaccharide side chains has no detectable effect on the surface-related 'peripheral-site' functions; like the wild-type enzyme, all mutants were inhibited by propidium and by increased concentrations of acetylthiocholine.

INTRODUCTION

Post-translational glycosylation can affect the biological activity of proteins, their transport towards the cell surface and the stabilization of their functional conformation (Chu et al., 1978; Gibson et al., 1979; Dube et al., 1988; Matzuk et al., 1989; Semenkovich et al., 1990). The acetylcholinesterases (AChE; EC 3.1.1.7), the main function of which is termination of acetylcholine-induced nerve impulse transmissions at cholinergic synapses, are multimeric glycosylated ectoenzymes. They are polymorphic in their quaternary structure (Massoulie and Bon, 1982; Chatonnet and Lockridge, 1989; Taylor, 1991) and, like the related butyrylcholinesterases (BuChE), carry various amounts of carbohydrate side chains attached to their core polypeptides (Liao et al., 1991, 1992; Treskatis et al., 1992). These carbohydrates are primarily asparagine-linked side chains, as indicated by their susceptibility to specific sugar hydrolases (Liao et al., 1992; Kronman et al., 1992) and by their selective affinity for lectins (Rotundo, 1988; Kerem et al., 1993).

Cholinesterase sequences display several potential N-glycosylation signals (Asn-X-Ser/Thr), but the number and location of these signals are not well conserved throughout the cholinesterase family. Human BuChE has nine potential sites (Lockridge et al., 1987; Prody et al., 1987), *Torpedo californica* AChE (TcAChE) has four sites (Schumacher et al., 1986) and the bovine enzyme has five sites (Doctor et al., 1990). The human AChE (HuAChE) sequence (Soreq et al., 1990), as well as the mouse and rat AChE (Rachinsky et al., 1990; Legay et al., 1993), display only three potential asparagine-linked glycosylation sites, located at positions 265 (258), 350 (343) and 464 (457) [number in parentheses corresponds to the analogous TcAChE position, according to nomenclature recommendations in Massoulie et al. (1992)]. These three N-glycosylation sites are conserved in all mammalian cholinesterases sequenced to date (Gentry and Doctor, 1991) and may represent a core of N-glycosylation targets required for their function.

The role of N-glycosylation in cholinesterases is not fully understood. In vertebrate cholinesterases, catalytic polypeptides with different glycosylation patterns have been isolated from different tissues (Bon et al., 1987; Liao et al., 1992; Treskatis et al., 1992; Campoy et al., 1992). In one case at least, this variation was associated with differences in catalytic properties (Meflah et al., 1984). In *Drosophila* AChE, on the other hand, mutagenesis of each one of the five potential N-glycosylation signals [none of which corresponds to sites conserved in mammalia (Hall and Spierer, 1986)] yielded enzyme molecules with wild-type activity (Mutero and Fournier, 1992).

Here we examine the role of the carbohydrate side chains in cholinesterase function by site-directed mutagenesis of recombinant HuAChE, employing an optimized expression system for production of secreted enzyme (Velan et al., 1991a; Kronman et al., 1992). This system is based on multipartite plasmids, carrying the human *ache* cDNA under control of the cytomegalovirus immediate early promoter enhancer, which are transfected into human embryonal 293 kidney cells. To allow for stable and continuous expression, the vectors carry the *neo* selector gene under the control of the H2L[4] promoter. The use of the low-efficiency promoter H2L[4] (Kronman et al., 1992) ensures selection of clones in which chromosomal integrations at transcriptionally active sites has occurred. These systems have been used to identify amino acid residues involved in catalytic activity, folding, post-translational processing and secretion of HuAChE (Shafferman et al., 1992a,b; Velan et al., 1991b; Kerem et al., 1993; Ordentlich et al., 1993). In the present study, we analyse

Abbreviations used: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; HuAChE, human AChE; TcAChE, *Torpedo californica* AChE; CAT/*cat*, chloramphenicol acetyltransferase.

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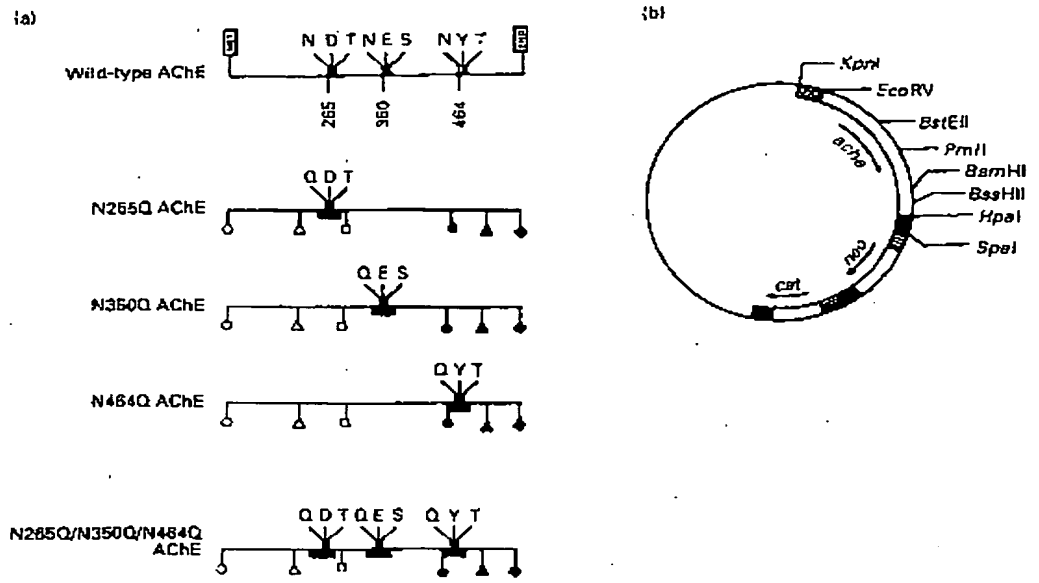

Figure 1 Strategy for generation of AChE mutant devoid of potential glycosylation sites

(a) N-glycosylation signals targetted for mutagenesis are presented in the top line (positions of asparagines are denoted), followed by three lines depicting single substitution mutants. Double (not shown) and triple (fourth line) substitution mutants were generated from these by ligating DNA fragments carrying the individual mutations. Unique restriction sites used for these constructions were *Eco*RV (○), *Bst*EII (△), *Pml*I (■), *Bam*HI (●), *Bss*HII (▲) and *Hpa*I (◆). (b) The prototype tripartite vector used for expression of wild-type and mutagenized recombinant HuAChEs. The three elements comprising the vector are: the *ache* gene controlled by the cytomegalovirus immediate early promoter/enhancer (grid); and *neo* gene controlled by the H2L^{d} histocompatibility antigen promoter (vertical stripes) and the *cat* gene controlled by the simian virus 40 early promoter/enhancer (horizontal stripes).

HuAChE polypeptides in which single or multiple N-glycosylation signals have been mutated, and examine the biosynthesis, stability and catalytic activity of the mutant enzymes.

EXPERIMENTAL

Construction of expression vectors for AChE and AChE mutants

Tripartite vectors (Figure 1b) (Shafferman et al., 1992a), expressing the human *ache* cDNA, the *cat* reporter gene (Gorman et al., 1982) and the selection marker *neo* (Southern and Berg, 1982), were generated as outlined previously (Kronman et al., 1992). Mutagenesis was performed by DNA cassette replacement into a series of HuAChE sequence variants which conserve the wild-type amino acids (Soreq et al., 1990), but carry new unique restriction sites based on the degeneracy of the genetic code. Detailed maps of the different human *ache* variants that express wild-type polypeptides are presented in Shafferman et al. (1992a).

The cDNA spanning the asparagine targetted for mutagenesis was excised by incision at the nearest restriction sites on the appropriate AChE cDNA variant and then replaced with a synthetic DNA duplex carrying the mutated codon. The N464Q mutant was generated by replacement of the *Bam*HI–*Bam*HI fragment of the native HuAChE cDNA. The distal *Bam*HI site was eliminated on ligation, as the GGG codon of Gly-487 was replaced by GGC in the synthetic DNA duplex. The N350Q mutant was generated by substituting the *Nar*I–*Bgl*II fragment of the human *ache* variant Ew5 (Shafferman et al., 1992a). The N265Q mutant was generated by replacement of an *Sal*I *Mlu*I DNA fragment in the *ache* variant Ew7 (Ew7 is a derivative of Ew5 in which new *Sal*I and *Mlu*I sites are engineered at codons 246/247 and 275/276 respectively). All asparagine codons in the putative N-glycosylation sites were replaced by the CAG codon of glycine. Synthetic DNA oligodeoxynucleotides were prepared using the automated Applied Biosystems DNA synthesizer, and sequences of all cloned synthetic DNAs were verified by the dideoxy sequencing method (USB sequenase kit).

Multiple substitution mutants were generated by cloning DNA fragments carrying the individual mutations into the tripartite vectors (Figure 1b). The DNA fragments used were: *Bst*EII–*Pml*I fragment for the N265Q substitution, *Pml*I–*Bam*HI fragment for the N350Q substitution and *Bam*HI–*Hpa*I DNA fragment for the N450Q substitution (Figure 1a).

Transfection of cells

CsCl-purified plasmid preparations were used to transfect human embryonal 293 kidney cells (Graham et al., 1977) employing the calcium phosphate method (Wigler et al., 1977). At least two different clone isolates were tested for each plasmid construct. Transient transfection was carried out as previously described (Velan et al., 1991a). After 24 h, cells were transferred to medium (2 ml per 100 mm plate) containing 10% AChE-depleted serum (Shafferman et al., 1992a) and incubated for 48 h. Medium was collected and assayed for AChE; medium of mock-transfected 293 cells served as control. Cell lysates were assayed for intracellular AChE and chloramphenicol acetyltransferase (CAT) activity (Gorman et al., 1982). Stably transfected isolated clones or pooled clones (about 50 clones per pool) were generated by G418 selected as previously described (Kronman et al., 1992).

Analysis of recombinant HuAChE mutants

AChE activity in medium of transfected cells was assayed by the method of Ellman et al. (1961). Standard assays were performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium

phosphate buffer, pH 8.0, 0.1 mg/ml BSA and 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27 °C and monitored by a Thermomax microplate reader (Molecular Devices). AChE protein mass was determined by e.l.i.s.a. based on polyclonal antibodies to native recombinant HuAChE (Shafferman et al., 1992a). This quantification was confirmed, whenever AChE quantities were sufficient, by immunoblots developed with conformation-independent anti-(recombinant HuAChE) antibodies (see below). Activity and AChE protein levels of the individual mutants (average of four transfections) were calculated by normalization to CAT activity as described in detail previously (Shafferman et al., 1992a). In mutants secreting low levels of AChE, cell growth medium samples were concentrated 10-fold by vacuum before quantification.

K_m values of the AChE mutants for acetylthiocholine were obtained from Lineweaver–Burk plots and k_{cat} calculations were based on the polyclonal e.l.i.s.a measurements. Interaction of the various mutants with AChE-specific inhibitors was monitored by comparing IC_{50} values with those of wild-type enzyme as described previously (Shafferman et al., 1992b). Both the peripheral anionic-site ligand, propidium, and the active-site inhibitor, edrophonium, were employed. In addition, we included the two bisquaternary inhibitors, decamethonium and BW284C51, which bridge the peripheral site and the active centre of the enzyme [for review, see Hucho et al. (1991)].

Sucrose-density-gradient centrifugation

Analytical sucrose-density-gradient centrifugation was performed on 5–25% sucrose gradients containing 50 mM sodium phosphate buffer, pH 8.0. Centrifugation was carried out in an SW41 Ti Rotor (Beckman) for 22 h at 160000 *g* (36000 rev./min) at 4 °C. Fractions of volume 0.3 ml were collected and assayed for AChE activity.

Visualization of AChE by SDS/PAGE

Recombinant HuAChE was immunoprecipitated from cell growth medium with rabbit anti-(recombinant HuAChE) antibodies bound to Sepharose as previously described (Kerem et al., 1993). Immunoadsorption was carried out for 2 h at room temperature, and immunoprecipitates were eluted from the Sepharose beads by 30 min incubation with Laemmli's sample buffer at room temperature. Immunoprecipitated AChE was subjected to SDS/PAGE (Laemmli, 1970) under reducing conditions and electroblotted on to nitrocellulose filters. Immunoblots were developed by immunostaining with rabbit antibodies directed to bacteria-derived HuAChE polypeptides (Velan et al., 1991b).

Metabolic labelling and immunoprecipitation of proteins

Cells were preincubated for 1 h in methionine-free Dulbecco's modified Eagle's medium supplemented with 10% AChE-depleted fetal calf serum. Cells were then labelled for 0.5 h with [^{35}S]methionine (200 μCi/ml; 1000 Ci/mmol; Amersham) and chased in methionine-containing medium supplemented with 20 μg/ml aprotinin (Sigma). After various chase periods, culture media were collected and cells were washed and then lysed by a modification of previously described procedures (Amitay et al., 1991; Weitz and Proia, 1992). Lysis buffer consisting of 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% sodium deoxycholate, 1% SDS, 1% Nonidet P40, 20 μg/ml aprotinin and 0.1 M iodoacetamide (freshly dissolved) was added to cells (1 ml of buffer, approx. 10^6 cells). Lysates were boiled for 5 min, cooled to room temperature, cleared by 15 min centrifugation at 16000 *g* and then diluted 1:4 in lysis buffer, to which 10 mg/ml BSA was added (iodoacetamide was omitted).

AChE was immunoprecipitated from cleared cell lysates and media with rabbit anti-(recombinant HuAChE) antibodies (Shafferman et al., 1992a) followed by precipitation with Protein A bacterial adsorbant (Biomakor) as described previously (Amitay et al., 1991).

RESULTS AND DISCUSSION

Generation of glycosylation-impaired AChE mutants

Each one of the three N-glycosylation signals of the HuAChE catalytic subunit was rendered non-functional by DNA-cassette replacement mutagenesis (Figure 1a). The respective asparagine codons were replaced by glycine codons to generate the three AChE mutants N265Q, N350Q and N464Q (CAG, the most commonly occurring glycine codon in HuAChE cDNA, was used in all replacements). These single mutants were then used to engineer the double mutants N265Q/N350Q, N265Q/N464Q and N350Q/N464Q and the triple mutant N265Q/N350Q/N464Q which is devoid of any potential N-glycosylation sites. All forms were integrated into multipartite expression vectors (Figure 1b) and used for transient or stable transfections of 293 cells.

The effect of elimination of potential N-glycosylation signals on glycosylation level was analysed by SDS/PAGE of secreted AChE mutant enzymes derived from isolated clones (see below) of stably transfected cells (Figure 2). Elimination of each individual glycosylation site at positions 265, 350 and 464 resulted in a decrease in the apparent molecular mass of the HuAChE polypeptide as visualized by blotting and immunostaining (Figure 2a). Accordingly, enzyme molecules with two mutations migrated faster than the single mutants, and the AChE subunit in which all three N-glycosylation signals were mutated migrated even faster (Figure 2b), forming a faint sharp band resembling the *N*-glycanase-treated wild-type AChE molecule (Kronman et al., 1992). These results indicate that all three Asn-Xaa-Ser signals of HuAChE are actually glycosylated, and agree with the three-dimensional model of HuAChE (Barak et al., 1992) which maps the three relevant asparagine moieties on the outer surface of the molecule (Figure 3). The migration patterns depicted in Figure 2 suggest that size heterogeneity characteristic of wild-type recombinant HuAChE (Kronman et al., 1992) originates from partial occupancy of one of the three glycosylation signals. The fast migrating minor band of the wild-type enzyme co-migrates with the major band of the single-site mutants, and the fast migrating band of the single-site mutants co-migrates with the major band of the double-site mutant. Comparison between migration patterns of the various mutants (Figure 2) may also suggest that the major target for this partial glycosylation is Asn-350, as only N350Q mutants lack the accompanying fast migrating minor band observed in the other single-site mutants.

Elimination of N-glycosylation sites did not interfere with the ability of AChE subunits to form thiol-linked dimers, as reflected in sucrose-density-gradient-sedimentation analysis (Figure 4). It appears therefore that the oligosaccharide side chains do not affect the structural elements that hold the two subunits of the AChE homodimer together. Indeed, none of the N-glycosylation sites is in close proximity (Barak et al., 1992) to helices αF'J and αH which form the four-helix bundle suggested by Sussman et al. (1991) to function in dimerization of TcAChE.

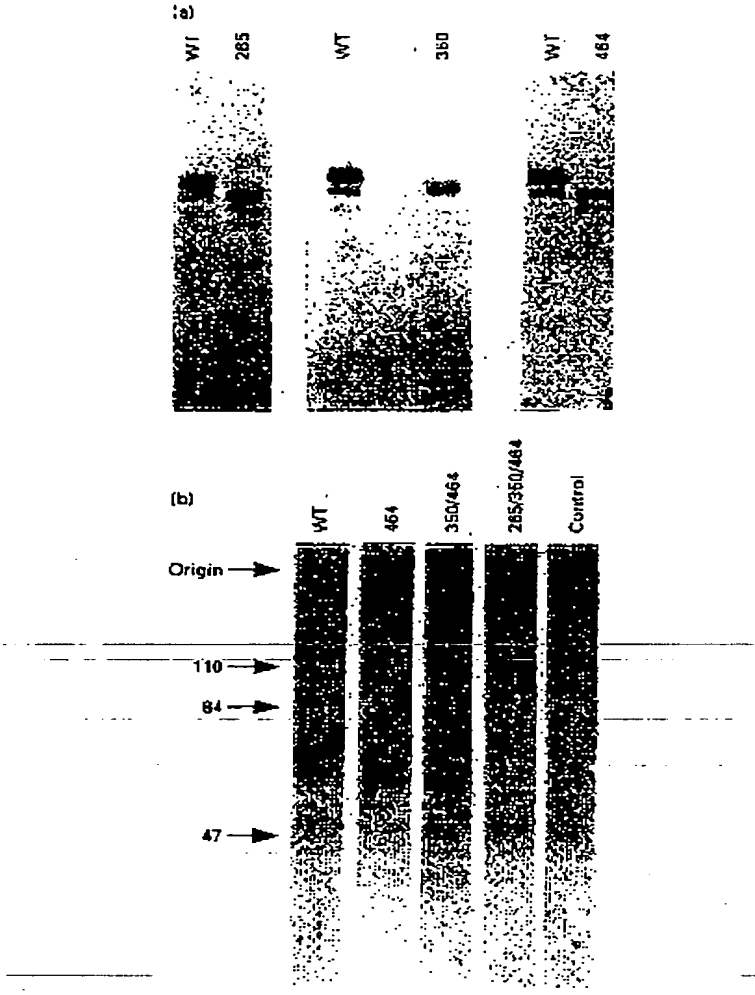

Figure 2 Effect of elimination of N-glycosylation signals on the electrophoretic mobility of recombinant HuAChE

Wild-type (WT) recombinant HuAChE and the various glycosylation-site mutants (designated by position of the mutated asparagines) were immunoprecipitated by specific antibodies from growth medium of stably transfected cell lines. Supernatant of non-transfected 293 cells served as control. Samples were obtained from clone pools or selected clones secreting 0.2–3 units/ml wild-type or mutant recombinant HuAChE. The relative amount of AChE in the various preparations is not a direct reflection of the production efficiency of the specific mutant, but rather of the production level of the individual cell clone used. Immunoprecipitates of individual single-site mutants (a) as well as multiple-site mutants (b) were subjected to SDS/PAGE under reducing conditions together with wild-type immunoprecipitates. AChE was electroblotted on to nitrocellulose and visualized by immunostaining as described in the Experimental section. Migration of prestained molecular mass markers (Bio-Rad marker kit) is indicated by arrows.

N-Glycosylation is required for efficient production of recombinant HuAChE by transfected cells

The effect of N-glycosylation on AChE production was examined in 293 cells transiently transfected with vectors expressing wild-type and mutant enzyme. Amounts of AChE secreted into the cell growth medium were monitored by activity measurements as well as by e.l.i.s.a. of AChE polypeptide mass [normalized to levels of the co-expressed CAT (Shafferman et al., 1992a)]. Results obtained by these two independent assay systems indicate that elimination of N-glycosylation sites has a pronounced effect on HuAChE levels secreted by the cells (Table 1). The decrease in yields of HuAChE was proportional to the number of available N-glycosylation sites. Elimination of a single site, two sites and all three sites resulted in secreted enzyme yields of 20–30%, 2–4% and about 0.5% respectively (Table 1). Thus N-glycosylation at each of the three sites appears to be important for efficient production of HuAChE by the recombinant 293 cells. One cannot overlook the possibility that the decrease in production reflects protein instability brought about by the Asn to Gln substitutions *per se*. However, when Asn-265 was substituted with a residue with minimal spatial requirements, namely alanine, the mutant (N265A) displayed the same phenotype as the N265Q mutant (80% decrease in enzyme secretion relative to the wild-type). Furthermore, we note that all three mutated asparagines map to the exposed surface of the folded polypeptide (Figure 3) and are probably not essential for maintaining the conformation (Alber et al., 1987; Wendell et al., 1992; Shafferman et al., 1992a).

The proposed influence of N-glycosylation on the production of recombinant HuAChE is in contrast with observations made with glycosylation-impaired *Drosophila* AChE in a *Xenopus* oocyte expression system (Mutero and Fournier, 1992). Nevertheless, recombinant HuAChE behaves like other eukaryotic glycoproteins, in which aberrations in post-translational glycosylation lead to impaired biosynthesis (Dorner et al., 1987; Machamer and Rose, 1988; Matzuk and Boime, 1988; Trifft et al., 1992; Weitz and Proia, 1992).

Secretion-incompetent molecules are formed in cells producing HuAChE glycosylation mutants

Analysis of the events leading to the low production of AChE by glycosylation-impaired mutants relied on intracellular chase of metabolically labelled molecules. To this end, we decided to generate stable cell clones expressing the various HuAChE N-glycosylation mutants. Whereas stable clones expressing the single-site glycosylation mutants were quite readily obtained, producer clones for multiple-site mutants could be established only for the double mutant (N350Q/N464Q).

Pulse–chase experiments performed with the stable clones indicate that glycosylation affects the secretion pathway of recombinant HuAChE. In the wild-type enzyme, the majority of the newly synthesized AChE polypeptides were secreted from the cells within 6 h, and were recovered efficiently in the medium (Figure 5a), in accordance with previous observations (Kerem et al., 1993). In the double-site mutant (N350Q/N464Q), only a small fraction of the labelled HuAChE molecules were secreted from the cells into the medium, whereas most of the newly synthesized mutant polypeptides were retained within the cells and could be recovered in lysates even 24 h after the pulse (Figure 5b). Retention of non-secreted AChE was also detected in cells expressing the single-site mutant N265Q, although in a smaller proportion than that observed for the double mutant.

Cell-associated AChE catalytic activity amounted to 6–8% of total activity produced within 24 h by cell cultures expressing the glycosylation-site mutant AChEs, irrespective of location or number of N-glycosylation-site mutations. This value was identical with that observed in cells expressing wild-type recombinant HuAChE. The accumulation of immunoreactive AChE suggested by the pulse–chase experiments (Figure 5) was not accompanied by a concomitant increase in cell-associated enzyme activity. Thus the AChE polypeptides retained in cells expressing the double mutant N350Q/N464Q appear to be non-active.

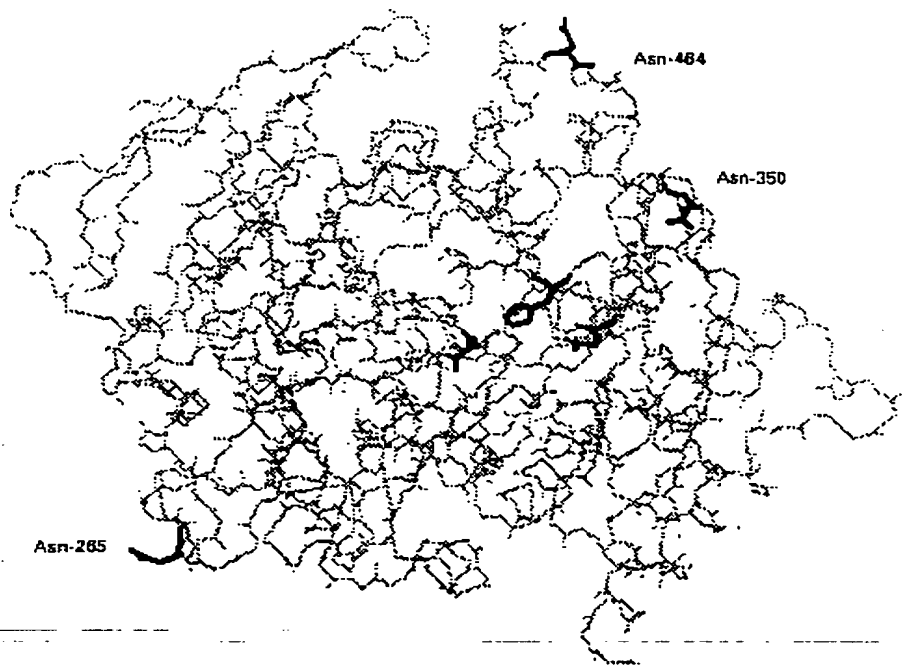

Figure 3—Mapping of the putative N-glycosylation targets on HuAChE

Three-dimensional carbon-α backbone trace of HuAChE, highlighting residues Asn-265, Asn 350 and Asn-464 as well as the active-site triad (in the centre). The three-dimensional model of HuAChE (Barak et al., 1992) is based on the structure of the *T. californica* enzyme (Sussman et al., 1991).

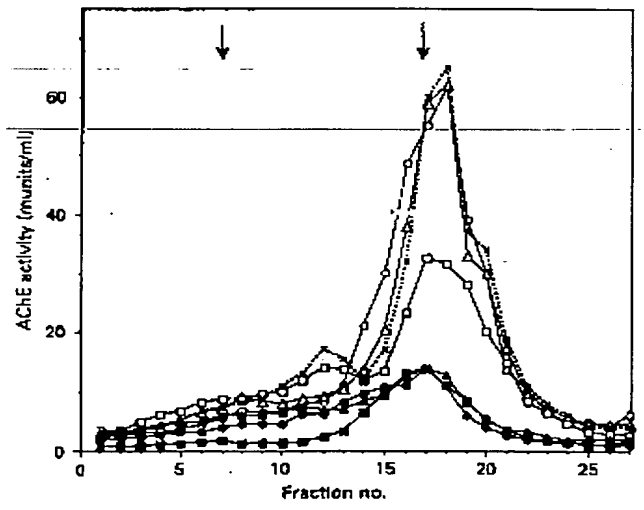

Figure 4 Sucrose-density-gradient profiles of HuAChE N-glycosylation mutants

Cell growth supernatants from 293 clone pools expressing wild-type AChE (0.2 ml/gradient) or mutant AChE (1 ml/gradient) were loaded on 5–25% sucrose gradients. The arrow on the left indicates sedimentation of β-galactosidase (16 S) and the arrow on the right indicates sedimentation of bovine intestinal alkaline phosphatase (6 S). HuAChE types analysed include: wild-type enzyme (X) and the mutants N350Q (○), N265Q (△), N464Q (□), N265Q/N350Q (●), N265Q/N464Q (▲) and N350Q/N464Q (■).

Table 1 Secretion levels of HuAChE by cells transfected with N-glycosylation-site mutants

HuAChE activity (munits) recovered from cell supernatant of a 100 mm culture plate 48 h after transfection is given. Values are CAT-normalized (Shafferman et al., 1992a) and represent the mean ± S.E.M. of quadruplicate transfections. HuAChE (ng) recovered from cell supernatant of a 100 mm culture plate 48 h after transfection was determined by e.l.i.s.a.; values are CAT-normalized as above.

	HuAChE activity		HuAChE production*	
HuAChE type	munits	% of wild-type	ng	% of wild-type
Wild-type	940 ± 110	100	128 ± 22	100
N265Q	160 ± 17	19	30 ± 4	23
N350Q	138 ± 18	14	32 ± 3	25
N464Q	235 ± 25	27	38 ± 5	29
N265Q/N464Q	17 ± 3	2	3 ± 1	2
N265Q/N350Q	18 ± 2	2	3.5 ± 1.5	3
N350Q/N464Q	17 ± 2	2	5 ± 2	4
N265Q/N350Q/N464Q*	3 ± 0.4	0.35	0.8 ± 0.4	0.6

* Values for the triple mutant were measured in 10-fold concentrated culture supernatant.

All these observations suggest that elimination of N-glycosylation sites leads to a less efficient folding of the nascent AChE polypeptides. In the double-site mutant N350Q/N464Q (Figure 5), where the fate of newly synthesized molecules could

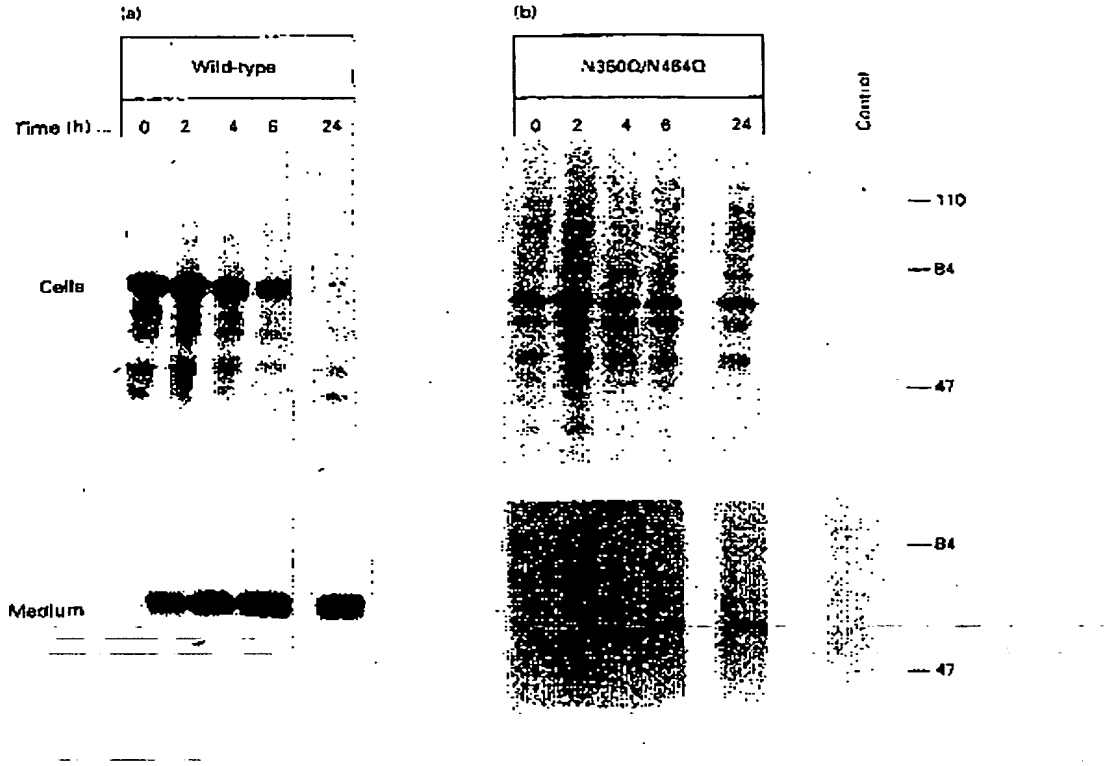

Figure 5 Time course for secretion of wild-type recombinant HuAChE and the N350Q/N464Q mutant

Cloned cells secreting approx. 30 units/ml wild-type (a) or approx. 1.5 units/ml N350Q/N464Q (b) HuAChE were pulse-labeled with [^{35}S]methionine for 30 min and chased for various times as indicated. Recombinant HuAChE was immunoprecipitated from solubilized cells and from medium. Non-transfected 293 cells served as control. Immunoprecipitated AChE was separated by SDS/PAGE under reducing conditions and visualized by fluorography. Exposure time of fluorographs was usually 2 days, but the fluorograph of N350Q/N464Q AChE secreted into the medium was exposed for 4 days. Positions of molecular-size markers (prestained Bio-Rad standards) are shown on the right (kb).

be followed, the majority of the AChE molecules appear to undergo aberrant folding into non-active secretion-incompetent forms, which are trapped in the cell. It is not clear whether the accumulated intracellular AChE is deleterious to the cells, but one could speculate that formation of intracellular non-active AChE pools may be responsible for difficulties in generating cell lines expressing other multiple-site glycosylation mutants, in spite of repeated attempts.

N-glycosylation contributes to HuAChE stability

The effect of N-glycosylation on thermostability of secreted HuAChE molecules was examined by comparing the time-dependent loss of activity at 55 °C in wild-type and mutant enzyme (Figure 6). The wild-type enzyme, as well as all mutants tested, exhibited a biphasic inactivation pattern (Edwards and Brimijoin, 1983; Velan et al., 1991b), which is characteristic of multimeric cholinesterases [the major population in each of the pooled-clone preparations used consists of dimeric forms (Figure 4)]. Loss of side-chain glycans affected AChE inactivation to different extents, but in all cases the effect on the first phase appears to be more pronounced than that on the second phase. Of the single-site mutants examined, the N350Q mutation had no effect on the heat-inactivation pattern, the N265Q mutation had a minor effect, and the N464Q mutation led to a significant decrease in thermostability (the half-life of the first phase is 2.2 min compared with 4.5 min for the wild-type). The double

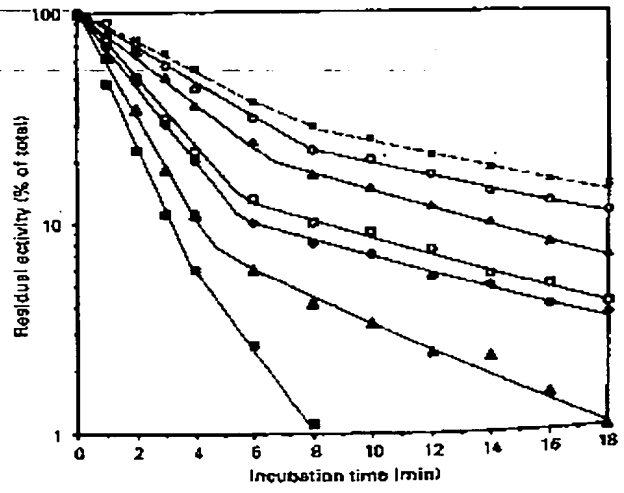

Figure 6 Thermal inactivation kinetics of wild-type and mutant HuAChEs

Portions of cell growth supernatant from stable clone pools, containing about 20 units/ml of wild-type or mutant AChEs, were incubated at 55 °C in the presence of 0.2 mg/ml BSA. At the indicated intervals, samples were chilled on ice and tested for residual activity. HuAChE forms analysed include: wild-type enzyme (X) and the mutants N350Q (○), N265Q (△), N265Q/N350Q (□), N265Q/N464Q (▲) and N350Q/N464Q (■).

mutants are more thermosensitive than the single mutants, and molecules that include the N464Q substitution are the most sensitive. The rank order of thermosensitivity among double mutants is N350Q/N464Q > N265Q/N464Q > N265Q/N350Q and the corresponding half-lives for the first component of the curve are 0.9, 1.2 and 2.0 min respectively. The thermosensitivity of the two double mutants carrying the N350Q substitution was greater than the sum of effects of the two corresponding single mutations. This synergism emphasizes the role of all the side-chain glycans in maintaining the mature enzyme in a thermodynamically stable configuration. Accurate heat-inactivation analysis of the triple-site mutant was prevented by the low production level of these molecules. It appears, however, that the triple mutant is less thermostable than any of the double mutants.

One is tempted to draw correlations between the role of glycans in generating stable secretion-compatible AChE configurations during the intracellular folding process and secretion, and their role in conferring resistance against thermal inactivation on the mature molecule. The overall N-glycosylation level evidently plays a key role in both processes, but correlations at the level of individual glycosylation sites cannot be found (for example the highly thermosensitive N464Q mutant is secreted at the same efficiency as other single-site mutants; compare Table 1 and Figure 6). This highlights the fact that the thermodynamic considerations for formation of the folded polypeptide and those for maintaining the stability of the folded polypeptide are not necessarily similar.

N-linked oligosaccharide side chains are not required for catalytic activity of recombinant HuAChE

In contrast with the significant effect on polypeptide biosynthesis, perturbation of N-glycosylation had no detectable effect on AChE catalytic activity of the secretion-compatible molecules. Specific activities calculated from the enzyme activities of the various glycosylation mutants and the respective e.l.i.s.a. values (Shafferman et al., 1992a) were found to be comparable with the wild-type value of about 6±2 munits/ng (Table 2). The fact that none of the mutants exhibited a higher apparent specific activity indicates that lack of glycosylation does not affect the efficiency of the e.l.i.s.a. (the bulk of polyclonal antibodies used in the assay probably interact with non-sugar epitopes).

Comparison of the catalytic constants for the hydrolysis of acetylthiocholine further establishes the similarity in the catalytic efficiency of the various glycosylation mutants. The K_m, $k_{cat.}$ and $k_{cat.}/K_m$ were not affected by any of the mutations (Table 2). Even HuAChE molecules devoid of all three glycosylation sites exhibited normal catalytic properties. Nevertheless, some differences between mutants were revealed when interaction with the active-site ligands was studied. AChE molecules carrying the N350Q substitution, alone or in combination with the other mutations, were less susceptible to inhibition by edrophonium, decamethonium and BW284C51 (Table 2). These results could indicate that the elimination of the oligosaccharide side chain attached to Asn-350 triggers a subtle conformational change at the active centre which affects interaction with the bulky inhibitor molecules. Interestingly, as noted above, a HuAChE glycoform lacking glycosylation at Asn-350 is probably generated during biosynthesis of the wild-type enzyme in recombinant cells (Figure 2).

Catalytic functions, in which the AChE peripheral anionic sites are believed to participate, were analysed by examining interactions with the selective ligand propidium as well as inhibition by increased concentration of substrate. All single-site and double-site mutants were inhibited by propidium at comparable efficiencies (Table 2) and exhibited the characteristic bell-shaped curve in response to high acetylthiocholine concentration (not shown). It appears therefore that the side-chain oligosaccharides do not affect surface-related AChE catalytic functions.

Taken together, these results indicate that N-glycosylation contributes to the secretion-compatible folding of the AChE polypeptide in the cell and to conformation stabilization thereafter. Nevertheless, glycosylation-deficient molecules that escape the secretion-related restrictions are secreted in a folded form that permits all the interactions pertinent to catalysis. This conclusion is also substantiated by reports on *in vitro* refolding of denatured non-glycosylated bacteria-derived recombinant cholinesterases into catalytically active polypeptides (Masson et al., 1992; Fisher et al., 1993).

Cholinesterases with different glycosylation patterns (probably generated through differential utilization of glycosylation sites or of biosynthetic pathways) have been isolated from various tissues of the same species. This was reported for *Electrophorus* AChE (Bon et al., 1987), bovine and human AChE (Meflah et al., 1984; Liao et al., 1991, 1992) as well as for chicken BuChE (Treskatis et al., 1992). Only in bovine leucocytes (Meflah et al., 1984) was the different glycosylation pattern associated with differences in enzymic properties.

The various recombinant HuAChE glycosylation variants generated here through elimination of N-glycosylation signals

Table 2 Catalytic functions of HuAChE and its mutants

Wild-type HuAChE values for edrophonium, propidium, decamethonium and BW284C51 are 7.5, 1.5, 8.5 and 0.008 μM respectively. Acetylthiocholine at 0.5 mM was used in inhibition studies. Results are means ± S.E.M. for four determinations. ND, not determined.

AChE type	Specific activity (munits/ng)	K_m (mM)	$10^5 \times k_{cat.}$ (min^{-1})	$10^3 \times k_{cat.}/K_m$ ($M^{-1} \cdot min^{-1}$)	IC_{50} (mutant)/IC_{50} (wild-type)			
					Propidium	Edrophonium	Decamethonium	BW284C51
Wild type	6.6 ± 2.2	0.14 ± 0.01	4.0 ± 1.0	29 ± 7	1	1	1	1
N265Q	5.3 ± 1.2	0.12 ± 0.01	4.4 ± 1.3	36 ± 11	1	1	1	1
N350Q	3.7 ± 0.9	0.14 ± 0.02	4.2 ± 0.8	30 ± 6	1	2.2	7.5	3
N464Q	6.2 ± 1.7	0.12 ± 0.01	3.6 ± 1.0	30 ± 9	1	1	1	1
N265Q/N464Q	5.3 ± 2.5	0.12 ± 0.01	3.7 ± 1.2	31 ± 10	1	-	1	1
N265Q/N350Q	4.4 ± 2.3	0.14 ± 0.02	4.0 ± 0.9	29 ± 7	1	1.8	7.5	3
N350Q/N464Q	3.3 ± 1.5	0.14 ± 0.01	3.9 ± 0.6	28 ± 4	1	2	7.5	2.9
N265Q/N350Q/N464Q	3.9 ± 2.5	0.15 ± 0.02	3.5 ± 1.0	23 ± 7	ND	ND	ND	ND

clearly exhibited very similar hydrolytic potentials. This was also true for recombinant HuAChE products in which additional N-glycosylation sites resembling those present in bovine AChE were engineered into the human enzyme (B. Velan, C. Kronman, A. Ordentlich and A. Shafferman, unpublished work). These observations suggest that differential glycosylation of cholinesterases *in vivo* is not involved in generation of variability in the catalytic profile, but is rather related to tissue-specific processes which control glycoprotein biosynthesis in different cells. It is still possible, however, that N-glycosylation has a specific role in the, as yet unsolved, non-cholinergic functions of cholinesterases in differentiation and development (Patinkin et al., 1990; Layer, 1992).

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